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The Evolution of Energy-Transducing Systems. Studies with Archaebacteria.

Semiannual Progress Report, March 1993 - August 1993

NASA Cooperative Agreement number: NCC 2-578[1)]

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Summary

N-ethylmaleimide (NEM) inhibits the ATPase of *H. saccharovorum* in a nucleotide protectable manner. The bulk of ^{14}C-NEM is incorporated into subunit I. Inhibition kinetics indicated a single binding site. To determine the sequence around this site, cyanogen bromide peptides of NEM-labeled ATPase enzyme were prepared and separated on Tris-Tricine gels. Autoradiography indicated that the NEM binding site is probably located in a fragment of M_r 10 - 12 K. This result will be confirmed by N-terminal sequencing of the peptide. Since the cysteinyl residue, to which NEM is bound, may be located at the C-terminal end, purification and proteolytic treatment of the 10 K peptide will be required.

One inhibitor of V-type ATPases, fluoresceinisothiocyanate (FITC) inhibited also the ATPase of *H. saccharovorum* . Preliminary results indicated protection against inhibition by nucleotides. Localization of the binding sited to the major subunits is in progress.

An extraction procedure for the membrane sector of the ATPase complex of *H. saccharovorum* yielded a preparation which was enriched in a peptide of Mr 5 500. Experiments to test the immunological crossreaction with subunit c from the *Escherichia coli* F-type ATPase and the labeling with ^{14}C-DCCD are currently carried out.

Polyclonal antiserum to the smaller of the major subunits of the ATPase from *H. saccharovorum* (subunit II) reacts in Western blots strongly with the α and β subunits of the F1 ATPase of *E. coli* , suggesting highly conserved regions on both types of ATPases. To elucidate further the regions of homology, cyanogen bromide peptides of the β subunit were prepared for sequence analysis.

[1]The NASA Technical Officer for this grant is Dr. L.I.Hochstein, NASA Ames Research Center, Moffett Field, CA 94035

[2]Abbreviations: DCCD, dicyclohexylcarbodiimide, NEM, N-ethylmaleimide, CB, cyanogen bromide

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Progress report

1. The ATPase from *Halobacterium saccharovorum* is inhibited in a nucleotide protectable manner by NEM[2)]and the bulk of the inhibitor is incorporated into subunit I (Ref.1). Using inhibition kinetics, we obtained evidence for one NEM binding site per molecule of enzyme. One goal of this Cooperative Agreement is the determination of the sequence around the NEM binding site. This information will allow a detailed comparison between the halobacterial ATPase and V-type ATPases. It has been proposed that archaebacterial ATPases are not likely to be inhibited by sulfhydryl reagents since they possess a serine instead of a cysteine in the conserved region GCGKT in the catalytic subunit (Ref.2). However, there are at least 3 cysteinyl residues in subunit I of the ATPase from *H. saccharovorum*, one of which is expected to be the NEM reactive residue.
The enzyme was labeled with ^{14}C-NEM and subunit I was prepared by electroelution. Subsequent cleavage with cyanogen bromide yielded approximately 10 - 12 peptides, which could be separated on Tris-Tricine gels, and which showed molecular masses below 18 kDa. This result was surprising insofar, as there should have appeared a fragment of about 23 kDa, assuming the sequence of subunit I from *H. halobium* (Ref. 3) is similar to that of *H. saccharovorum* . Autoradiography of the gels containing the peptides showed the bulk of radioactivity associated with a peptide of M_r of ca. 10 - 12 000. The N-terminus of this peptide will be sequenced to confirm the location. If correct, and again assuming that the sequence of *H. halobium* is for the most part identical to that of *H. saccharovorum* , the reactive cysteinyl residue will be at the C-terminal end. Therefore, the peptide will be purified by electroelution, treated with proteases (chymotrypsin or V8 protease) and peptides separated on a reverse phase column. There is likely to be only one radioactive peptide, which will be sequenced.

2. A recently identified inhibitor of plant vacuolar ATPases is fluorescein isothiocyanate (FITC). Membranes and solubilized ATPase from *H. saccharovorum* were treated with FITC. Concentrations of 1 - 2 mM caused an inhibition of hydrolytic acticity by about 50 %. Preliminary data indicated protection against inhibition by nucleotides. The location of the FITC to the ATPase subunits will be determined by SDS gel electrophoresis

and subsequent illumination with long wave ultraviolet light.
3. The membrane portion of the halobacterial ATPase has not been characterized yet. A commonly used procedure for the extraction of the so-called proteolipid (subunit c in *E. coli*) is treatment with chloroform-methanol (2:1). When applied to membranes from *H. saccharovorum*, this method yielded a preparation which was enriched in a protein of Mr 5 500. With an existing antiserum to subunit c, the immunoreactivity of the extract will be probed. In addition, the proteolipid extract will be prepared following labeling with of membranes with ^{14}C-DCCD.

4. Polyclonal antibodies to both of the major subunits of the halobacterial ATPase reacted also with the major subunits of the V-ATPase of *Neurospora crassa*, confirming the relationship between these two enzymes. A strong crossreaction of the antiserum to subunit II was found with the β subunit of the *E. coli* F-ATPase (Ref.4). Several cyanogen bromide fragments of the β subunit still reacted with the antiserum. This suggested highly conserved regions present on both types of ATPases, which might be relics of a common progenitor ATPase. Two of the reactive CB peptides from the β subunit were prepared and submitted for sequencing analysis. It is expected that a localization of the conserved regions to proposed nucleotide binding sites (or portions thereof) should be possible.

Personnel

The described work was carried out by the P.I., lab assistant E. Emrich and two graduate students (S. Hager and C. Radax) from the University of Vienna, Austria.

Other Activities

The characterization of halophilic isolates from salt sediments was continued in collaboration with laboratories in England (W. D. Grant), Germany (G. Wanner) and Austria (E. Denner, J. Busse). Results from this work were presented in a poster at the BioAstronmy meeting in Santa Cruz, August 1993 (Ref.5) and will be reported in a paper (in preparation).
March 93 - July 93: Teaching appointment at the University of Vienna,

Austria, while remaining at reduced time (10%) with the SETI Institute.

References:

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